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Method Development and Validation Study for Quantitative Determination of 3-Ethynylaniline Content in Erlotinib by Liquid Chromatography–Tandem Mass Spectrometry

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ABSTRACT

A sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed for the quantitative determination of 3-ethynylaniline, a process related genotoxic impurity in synthesis of erlotinib. The 3-ethynylaniline in erlotinib drug was analyzed on a Hypersil BDS C-8 (50 mm × 4.6 mm, 3 µm) column interfaced with a triple quadruple tandem MS operated in a multiple reaction monitoring mode. Positive electrospray ionization was employed as the ionization source and the mobile phase used was 5.0 mM ammonium acetate–acetonitrile (55:45, v/v). The developed method was validated as per ICH guidelines in terms of specificity, limit of detection, limit of quantification, linearity, precision, accuracy, and robustness. This method shows excellent sensitivity in the quantification of impurity at the level of 0.6 ppm with 1.9% relative standard deviation. The calibration curve showed good linearity over the concentration range of 0.6-6.0 ppm with a correlation coefficient of >0.9991. The accuracy of the method was between 96.26% and 100.64%.

Key words: *Erlotinib, Genotoxic impurity, Liquid chromatography–tandem mass spectrometry, Method development, Validation.*

1. INTRODUCTION

The presence of genotoxic impurities in active pharmaceutical ingredient (API) and products of drugs have recently drawn more attention by regulatory authorities and pharmaceutical industries as they have exceedingly destructive effects on human health. Even lower concentration (<1 ppm) of genotoxic impurities also causes genetic mutations, chromosome breaks and leads to oncological diseases. In view of the current situation, regulations defining the approaches to control of genotoxic impurities in drugs need to be developed [1]. The European Medicines Agency (EMA) was one of the first regulators to develop the basic principles for evaluating genotoxic impurities in drugs [1]. The FDA published draft guidelines at the end of 2008 for manufacturers and industries on genotoxic and carcinogenic impurities in drug substances and products [2]. The ICH M7 guideline, in contrast with those of FDA and EMA, was applicable not only for the assessment of drug impurities that were reported for registration and clinical trials but also for re-examination of requirements for already registered drugs such as process change for a drug substance or drug production and use of new highly sensitive analytical methods [3]. In general, the sources of organic genotoxic impurities in drugs are starting materials, side products, intermediates, degradation products, and reagents of the production of drug. As per the recommendations of ICH, there is a need to remove the impurities or to reduce their content to a safe level. As per the guidelines of ICH M7, FDA and EMA the acceptable level of a genotoxic impurities is calculated based on the toxicological concern and is considered safe for lifetime use is 1.5 µg day^{-1} [1-3].

Erlotinib hydrochloride is used for the treatment of non-small cell lung malignancy, pancreatic cancer and several other types of cancer [4]. Its IUPAC name is N-(3-ethynylphenyl)-6, 7-bis (2-methoxyethoxy) quinazolin-4-amine hydrochloride. Erlotinib hydrochloride is synthesized from 4-chloro-6,7-bis (2-methoxyethoxy)-quinazoline and 3-ethynylaniline in dimethylformamide [5]. The genotoxicity of the 3-ethynylaniline is unknown but contain an alerting functional moiety [6]. Hence, 3-ethynylaniline compound is treated as potential genotoxic impurity.

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The structures of 3-ethynylaniline and erlotinib are given in Figure 1.

Regulatory authorities may be expected to control the levels of 3-ethynylaniline to be 10.0 ppm in the drug substance (assuming a 1.5 μg day^{-1} daily dose). Development of a method for the determination of genotoxic impurities at lower level using conventional analytical instruments (high-performance liquid chromatography [HPLC], gas chromatography, ultraviolet [UV]-visible spectrophotometry) is great challenge in the pharmaceutical industry as the sensitivity of these instruments is low. Hence, the sensitivity of analytical equipment must be increased in order to determine impurities at very lower levels (≤1-5 ppm). This can be achieved by combining HPLC with mass spectroscopy (MS). Because of reliability and high sensitivity of liquid chromatography (LC)/MS/MS method, recently so many authors used this method for the determination of organic genotoxic impurities [7-10]. So in this work, LC/MS/MS method was used for the determination of 3-ethynyl aniline in erlotinib.

The literature survey revealed that some spectrophotometric methods, HPLC methods, and LC/MS/MS were developed for the determination of erlotinib in different combinations of drugs and biological matrices [11-24]. But no method has been developed fort the determination of 3-ethynyl aniline at ultra-level (0.6 ppm) in erlotinib hydrochloride. Hence, this study was undertaken to develop a sensitive and rapid LC/MS/MS method for the determination of 3-ethynylaniline in erlotinib. Due to its higher selectivity and sensitivity, LC/MS/MS has been adopted for quantification of 3-ethynylaniline in erlotinib.

2. EXPERIMENTAL

2.1. Materials

HPLC grade acetonitrile and ammonium acetate were supplied by Merck (Mumbai, India). analytical grade of formic acid, trifluoroacetic acid, and methanol were obtained from SD Fine Chemicals Limited (Mumbai, India). Milli-Q-Plus (Millipore, Milford, MA, USA) water purification system was used for purified water. Reference standards of 3-ethynylaniline were obtained from Sigma-Aldrich (St. Louis, MA, USA). Nylon filter membranes (0.22 μm × 47 mm dia.) were purchased from Fisher Scientific Pvt. Ltd., (Mumbai, India).

2.2. Sample and Standard Preparation

A stock solution (0.1 mg mL^{-1}) of 3-ethynylaniline was prepared by taking of 10 mg of impurity in 100 ml of methanol. Further diluted stock solution (0.001 mg mL^{-1}) was prepared by diluting 1.0 ml of the above solution into 100 ml with methanol. The sample solution was prepared by dissolving 20 mg of accurately weighed erlotinib into a 10 ml of methanol. Finally, 0.0002 mg mL^{-1} (10 ppm) of 3-ethynylaniline was prepared with respect to erlotinib sample concentration (2 mg mL^{-1}). The potential genotoxic impurity samples for validation at 0.2, 0.6, 4.0, 6.0, 8.0, 10.0 and 12.0 ppm concentrations relative to the drug substance were prepared in the same manner using diluted stock solution. The concentration of the standard solutions and samples was optimized to achieve a desired signal-to-noise ratio (S/N) and good peak shape. All the standards were sonicated well and then filtered through 0.22 μm membrane filters before analysis.

2.3. Instrumentation

The MS of LC/MS/MS system used was an Applied Biosystems Sciex API 4000 model (Switzerland). The HPLC consisting of LC-20AD binary gradient pump, a SPD-5AVP UV detector, SIL-5HTC autosampler and a column oven CTO-5ASVP (Shimadzu Corporation, Kyoto, Japan) was used for method development and validation. Data acquisition and processing were conducted using the Analyst 1.5.1 software on a dell computer (Digital Equipment Co.).

2.4. Operating Conditions of LC/MS/MS

The analytical column used in LC/MS/MS was Hypersil BDS C8 (50 mm × 4.6 mm) 3 μm column (Thermo Co., USA) in isocratic mode using 5 mM ammonium acetate and acetonitrile in the ratio of 55:45 (v/v). The flow rate was 0.8 ml min^{-1} with the flow rate split down to 0.2 ml min^{-1} into the MS source. The column oven temperature was maintained at 40°C,

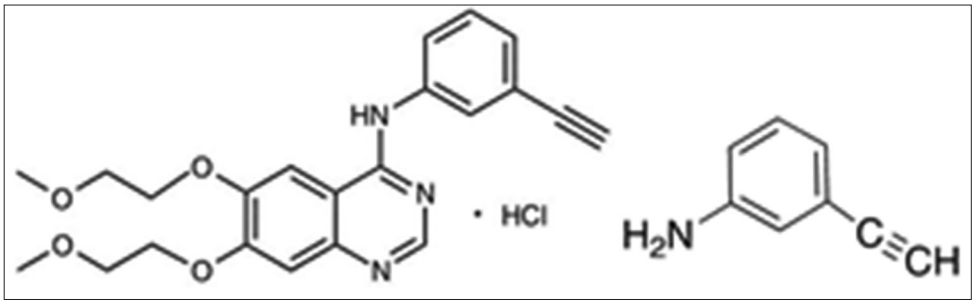

Figure 1: Structures of erlotinib hydrochloride and 3-ethynyl aniline.

sample cooler temperature was 5°C. The injection volume was 5 µl. Positive ion electrospray ionization probe was run with multiple reaction monitoring mode was used as MS method for quantification of 3-ethynylaniline in erlotinib drug substance. In this method, 3-ethynylaniline was monitored with its molecular ion $[M+H]^+$ m/z 118.2 (protonated) and daughter ion m/z 91.1 (118.2-91.1) and erlotinib was monitored with its molecular ion $[M+H]^+$ m/z 394.2 (protonated). The ion spray voltage (V), declustering potential and entrance potential were kept as 4500 V, 50 V and 5 V, respectively. The ion source gas1 and ion source gas pressure (psi) were maintained as 35 and 30, respectively. Collision energy is used 20 V.

3. RESULTS AND DISCUSSION

3.1. Method Development

The main aim of the LC/MS/MS method in this study was to separate and quantify 3-ethynyl aniline impurity with erlotinib API. Different makes of C-18 columns are used to separate 3-ethynylaniline impurity from erlotinib, but the resolution was not good. To get the better separation and sensitivity ammonium acetate used as neutral buffer and modified with acetonitrile as modifier. Chromatographic separation was finally achieved on a Hypersil BDS C8 (50 mm × 4.6 mm) 3 µm column (Thermo Co, USA) in isocratic mode using 5 mM ammonium acetate and acetonitrile in the ratio of 55:45 (v/v). The flow rate was 0.8 ml min^{-1}, with the flow rate split down to 0.2 ml min^{-1} into the MS source.

3.2. Method Validation

3.2.1. Specificity

Erlotinib and 3-ethynylaniline solutions were prepared individually at a concentration of about 0.01 mg mL^{-1} in the diluent, and a solution of erlotinib spiked with 3-ethynylaniline was also prepared. Specificity of this method was verified by injecting blank, erlotinib, 3-ethynylaniline and spiked samples separately, where no interference was observed at retention time of 3-ethynylaniline. Specificity chromatograms are shown in Figures 2-4.

3.2.2. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ values of 3-ethynylaniline were calculated from S/N ratios. Concentrations of solutions were reduced sequentially such that they yield S/N ratio as 2.8 and 9.9 for 3-ethynylaniline. The determined LOD and LOQ chromatograms were shown in Figures 5 and 6. % Relative standard deviation (RSD) of data generated from six injections of 3-ethynylaniline (without API) at LOQ level was 1.9%.

3.2.3.Linearity with LC/MS/MS

The linearity of 3-ethynylaniline was satisfactorily demonstrated with a six-point calibration graph between LOQ to 120% of analyte concentrations

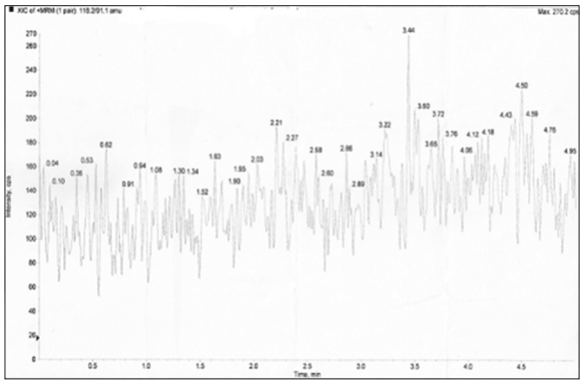

Figure 2: Blank chromatogram.

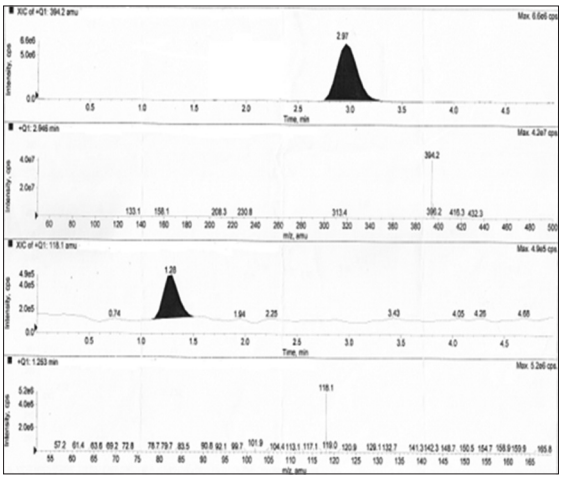

Figure 3: Chromatograms of erlotinib (above) and 3-ethynylaniline (below) along with mass spectra.

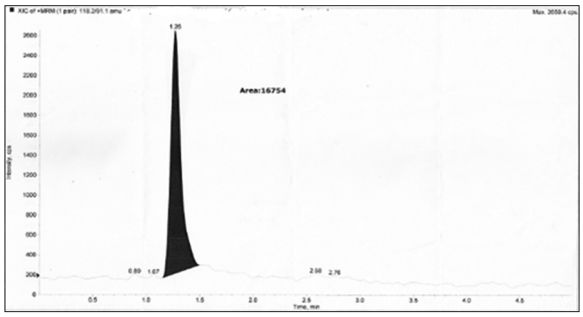

Figure 4: Spiked chromatogram of 3-ethynylaniline at limit of quantification level.

(LOQ, 40, 60, 80, 100 and 120%). The calibration curve was produced by plotting the peak areas of 3-ethynylaniline injections against its concentrations. The slope, intercept and correlation coefficient values were derived from linear least-square regression analysis, and the data are presented in Table 1. It reveals that an excellent correlation exists between the peak areas and concentrations of 3-ethynylaniline.

3.2.4. Recovery studies

Recovery studies were performed by the standard addition method to evaluate accuracy. The accuracy

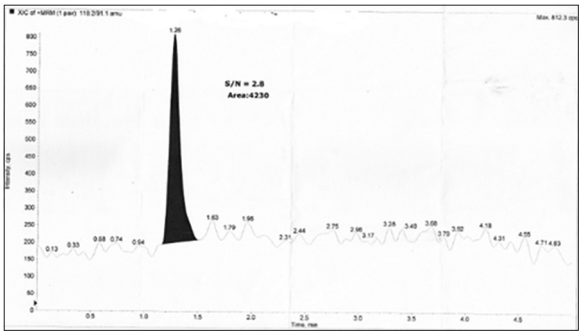

Figure 5: Chromatogram of 3-ethynylaniline at limit of detection level.

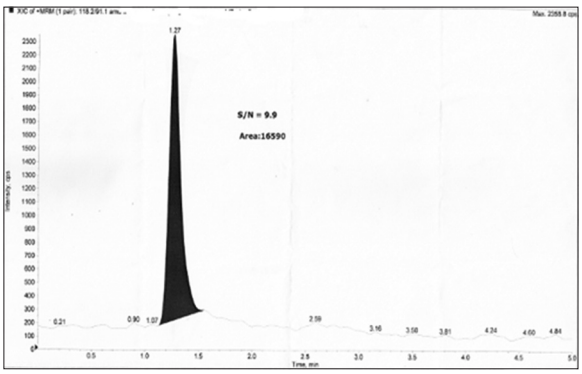

Figure 6: Chromatogram of 3-ethynylaniline at limit of quantification level.

of the method was determined in triplicate at LOQ level in bulk drug sample. Then the percentage recoveries were calculated. Excellent recovery values of 3-ethynylaniline (96.26-100.64) was obtained. At such a low levels these recoveries and % RSD is <2% was satisfactory. % RSD were calculated from the peak areas of 3-ethynylaniline and the results are tabulated in Table 2.

3.2.5. Robustness

The robustness of the method was studied with deliberate modifications in flow rate of mobile phase and column temperature. The flow rate of mobile phase was altered by 0.08 units, i.e. 0.72-0.88 ml min^{-1}. The effect of column temperature on the resolution was studied at 38°C and 42°C (temperature altered by 2 units), the results revealed that these changes do not impact on chromatographic performance.

3.2.6. Solution stability

The stability experiments were performed thoroughly to evaluate the stability of 3-ethynyl aniline standard solutions and spiked solutions at room temperature. The difference between recoveries at 0^{th} h and 24^{th} h was not more than 10%, which indicates that the standard prepared in diluent was stable more than 24 h.

4. CONCLUSION

A rapid, sensitive and specific LC/MS/MS method has been developed for the determination of 3-ethynylaniline genotoxic impurity. The method was validated for accuracy, precision, linearity, robustness, and stability. This analytical method is capable of quantifying 3-ethynylaniline at 0.6 ppm with respect to the concentration of 2 mg mL^{-1} of erlotinib drug substance. The method was fully validated and presents good linearity, specificity, accuracy, precision, and robustness. The LOD and LOQ values for 3-ethynylaniline are very low as 0.2 and 0.6 ppm, respectively. The sample prepared in analytical solution is found to be stable for 24 h. The method presented here could be very useful for monitoring of 3-ethynylaniline in erlotinib during its manufacturing as well as API.

Table 1: Linearity results.

% Level	Concentration (ppm)	Peak area
LOQ	0.60	16,520
40	4.00	55,452
60	6.00	164,255
80	8.00	218,146
100	10.00	286,642
120	12.00	335,164
	Correlation	0.9991
	Slope	28,162.4
	Intercept	−3457.06

LOQ=Limit of quantification

Table 2: Recovery results.

% Level	Theoretical concentrated	Measured concentrated	% Recovery	% RSD
LOQ	0.60	0.6038	100.64	1.00
	0.60	0.5919	98.65	
	0.60	0.6003	100.05	
50	5.00	4.8621	97.25	0.90
	5.00	4.9163	98.33	
	5.00	4.8300	96.61	
100	10.00	9.9190	99.20	1.30
	10.00	9.9559	99.56	
	10.00	9.7101	97.11	
150	15.00	14.4383	96.26	1.7
	15.00	14.9407	99.61	
	15.00	14.7500	98.34	

Concentrations are in ppm. RSD=Relative standard deviation, LOQ=Limit of quantification

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***Bibliographical Sketch**

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